Published in final edited form as:

Bone. 2015 March ; 0: 137–147. doi:10.1016/j.bone.2014.11.017.

Improvement of the skeletal and dental hypophosphatasia phenotype in *Alpl*^{−/−} mice by administration of soluble (non-targeted) chimeric alkaline phosphatase

Kellen Gasque^{a,1}, Brian L. Foster^{b,1}, Pia Kuss^{a,1}, Manisha C. Yadav^a, Jin Liu^c, Tina Kiffer-Moreira^a, Andrea van Elsas^d, Nan Hatch^c, Martha J. Somerman^b, and JoséLuis Millán^{a,*}

^aSanford Children's Health Research Center, Sanford-Burnham Medical Research Institute, La Jolla, CA 92037, USA ^bLaboratory of Oral Connective Tissue Biology, National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health, Bethesda, MD, USA ^cDepartment of Orthodontics and Pediatric Dentistry, School of Dentistry, University of Michigan, Ann Arbor, MI, USA ^dAM-Pharma B.V., Bunnik, The Netherlands

Abstract

Hypophosphatasia (HPP) results from *ALPL* gene mutations, which lead to a deficiency of tissue-nonspecific alkaline phosphatase (TNAP), and accumulation of inorganic pyrophosphate, a potent inhibitor of mineralization that is also a natural substrate of TNAP, in the extracellular space. HPP causes mineralization disorders including soft bones (rickets or osteomalacia) and defects in teeth and periodontal tissues. Enzyme replacement therapy using mineral-targeting recombinant TNAP has proven effective in preventing skeletal and dental defects in TNAP knockout (*Alpl*^{−/−}) mice, a model for life-threatening HPP. Here, we show that the administration of a soluble, intestinal-like chimeric alkaline phosphatase (ChimAP) improves the manifestations of HPP in *Alpl*^{−/−} mice. Mice received daily subcutaneous injections of ChimAP at doses of 1, 8 or 16 mg/kg, from birth for up to 53 days. Lifespan and body weight of *Alpl*^{−/−} mice were normalized, and vitamin B6-associated seizures were absent with 16 mg/kg/day of ChimAP. Radiographs, μ CT and histological analyses documented improved mineralization in cortical and trabecular bone and secondary ossification centers in long bones of ChimAP16-treated mice. There was no evidence of craniosynostosis in the ChimAP16-treated mice and we did not detect ectopic calcification by radiography and histology in the aortas, stomachs, kidneys or lungs in any of the treatment groups. Molar tooth development and function improved with the highest ChimAP dose, including enamel, dentin, and tooth morphology. Cementum remained deficient and alveolar bone mineralization was reduced compared to controls, though ChimAP-treated *Alpl*^{−/−} mice featured periodontal attachment and retained teeth. This study provides the first evidence for the

© 2014 Published by Elsevier Inc.

*Corresponding author. millan@sanfordburnham.org (J.L. Millán).

¹All three authors contributed equally.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bone.2014.11.017>.

Uncited references

[7,14,20,25,32,47]

pharmacological efficacy of ChimAP for use in the treatment of skeletal and dental manifestations of HPP.

Keywords

Enzyme replacement; Hypophosphatasia; Rickets; Osteomalacia; Craniosynostosis; Seizures

Introduction

Hypophosphatasia (HPP) is an inborn-error-of-metabolism caused by loss-of-function mutations in the *ALPL* gene located on chromosome 1 [17,43], which encodes the tissue-nonspecific of alkaline phosphatase (TNAP), also known as liver–bone–kidney type alkaline phosphatase (AP) [30,45]. The other three human APs are the placental (PLAP), germ cell (GCAP) and intestinal (IAP) isozymes encoded by syntenic genes (*ALPP*, *ALPPL2* and *ALPI*, respectively) found clustered on chromosome 2 [30]. HPP is characterized by subnormal plasma TNAP activity, leading to accumulation of the physiological substrates inorganic pyrophosphate (PP_i), a potent mineralization inhibitor [27], and pyridoxal-5'-phosphate (PLP), the major circulating form of vitamin B6 [35,42,45]. These biochemical changes underlie the most significant symptomatology in individuals with HPP: accumulation of PP_i resulting in rickets in children or osteomalacia in adults [45], as well as dental defects [15,16,38]. The inability to produce pyridoxal (PL) from PLP leads to defective production of neurotransmitters such as γ-aminobutyric acid (GABA) and serotonin, leading to seizures in the most severely affected patients [3,35,42].

The clinical severity of HPP is broad-ranging, encompassing stillbirth with complete absence of mineralized bones to defects limited to teeth and supporting structures, as in the odonto-HPP form [45]. To-date, 280 different *ALPL* mutations have been found associated with HPP, about 70% of which are missense mutations (http://www.sesep.uvsq.fr/03_hypo_mutations.php). Compound heterozygosity of severe recessive *ALPL* alleles are most often associated with life-threatening HPP disease [17,34,55], while the mild forms of HPP mostly result from dominant negative effects of severe alleles or from compound heterozygosity for severe and moderate alleles [9]. TNAP knockout mice (*Alpl*^{−/−}) phenocopy the infantile form of HPP extremely well [10,36]. *Alpl*^{−/−} mice are born with a normal skeleton, but develop rickets starting from postnatal day 6 and die before weaning with severe skeletal disease, following acute episodes of apnea and seizures [1,2,10,18,35,36].

To date, there is no established medical treatment for HPP [45]. Attempted enzyme replacement therapy (ERT) using intravenous (IV) infusions of ALP-rich plasma from Paget's bone disease patients, purified human liver ALP, or purified human placental ALP failed to rescue severely affected infants [44,48–50]. From these studies, it was concluded that TNAP activity might need to be directed to the skeleton rather than simply enhanced in the circulation in order to prevent or reverse the pathophysiology of HPP [5,46]. This hypothesis was supported by improvement in two unrelated girls with infantile HPP, following transplantation of healthy mesenchyme-derived cells in the skeleton [5,46]. Thus,

scientists from Enobia Pharma (Montreal, Canada) developed a mineral-targeted form of recombinant TNAP (sALP-Fc-D₁₀, aka as ENB-0040 or asfotase alfa) and our group administered it subcutaneously to *Alpl*^{-/-} mice from birth [31]. The treated *Alpl*^{-/-} mice grew normally, appeared well and showed no evidence of epilepsy or skeletal mineralization defects [31,52]. The first clinical study using asfotase alfa in patients with life-threatening HPP disease has also reported skeletal improvement and prevention of seizures in patients diagnosed with life-threatening HPP [51]. This treatment also prevented dental defects in *Alpl*^{-/-} mice, preserving acellular cementum [28], dentin [13] and enamel mineralization [53], demonstrating the robust nature of this mineral-targeting enzyme replacement therapy.

However, we and others have also shown that the sustained availability of soluble (non-targeted) TNAP, introduced either via an adeno-associated viral vector [26] or via direct injection of recombinant soluble TNAP (Oikawa et al., 2013) can prolong life, prevent seizures and improve the skeletal phenotype of *Alpl*^{-/-} mice. In this paper, we report studies using a human chimeric recombinant alkaline phosphatase, ChimAP, generated by substituting the flexible crown domain of a human IAP with that of a human PLAP isozyme, a substitution that preserves the three-dimensional structure and dimeric configuration of the recombinant enzyme [21]. ChimAP has catalytic properties distinct from those of recombinant TNAP, and has been developed for therapeutic use in acute kidney injury due to its increased stability, increased Zn²⁺ binding affinity, increased transphosphorylation, a higher turnover number and narrower substrate specificity with selectivity for bacterial-derived lipo-polysaccharides (LPS) compared to the parent IAP isozyme or to TNAP [21]. Here we show remarkable efficacy of daily administration of soluble (non-targeted) ChimAP in prolonging life, preventing seizures and ameliorating skeletal and dental disease in *Alpl*^{-/-} mice. We discuss the potential therapeutic applications of this ChimAP in HPP.

Materials and methods

Mice

The generation and characterization of the *Alpl*^{-/-} mice has been reported previously [36]. *Alpl*^{-/-} mice phenocopy infantile HPP, including global deficiency of TNAP, PP_i accumulation and mineralization defects [1,10,31,35]. Dietary supplementation with vitamin B6 briefly suppresses seizures and extends lifespan until postnatal days 18–22 but hypomineralization and accumulation of osteoid continued to worsen with age [10,31,35,36]. Therefore, all animals (breeders, nursing mothers, pups, and weanlings) in this study were given free access to modified laboratory rodent diet 5001 containing increased levels (325 ppm) of pyridoxine. Genotyping was performed by PCR on genomic DNA as previously described [52]. The Institutional Animal Care and Use Committee (IACUC) approved all animal studies.

Soluble chimeric human alkaline phosphatase (ChimAP)

The chimeric enzyme was generated by substituting the flexible crown domain of a human intestinal alkaline phosphatase (IAP) with that of a human placental alkaline phosphatase (PLAP) isozyme [21]. ChimAP retains the structural folding and dimeric configuration of IAP, but displays greatly increased stability, active site Zn²⁺ binding, increased

transphosphorylation, a higher turnover number and narrower substrate specificity, with comparable selectivity for bacterial LPS. Regarding PP_i hydrolysis, ChimAP has a ten-fold higher K_m and similar k_{cat} values compared to TNAP [21]. A solution of ChimAP at 10.1 mg/ml was provided by AM-Pharma (Bunnik, The Netherlands) in 25% glycerol w/v, 5 mM Tris/HCl, 2 mM MgCl₂, 50 μ M ZnCl₂, and at pH 8.0. The enzyme had a purity of >99.99% as determined by high-pressure liquid chromatography.

Dose–response study with ChimAP

Alpl^{−/−} mice were divided into 5 cohorts: Vehicle-treated: *Alpl*^{−/−} mice treated with vehicle (n = 14) only; ChimAP1: *Alpl*^{−/−} mice treated with ChimAP at 1 mg/kg/day (n = 14); ChimAP8: *Alpl*^{−/−} mice treated with ChimAP at 8 mg/kg/day (n = 12); and ChimAP16: *Alpl*^{−/−} mice treated with ChimAP at 16 mg/kg/day (n = 10). Wild-type littermates of *Alpl*^{−/−} mice served as reference animals and did not receive injections (n = 14). The vehicle or ChimAP cohorts were injected daily SC into the scapular region. Injections were administered between 8:00 and 11:00 AM. Volumes administered were calculated based on body weight measured prior to injection. All treatments began on postnatal day 1, and were repeated daily for up to 53 days or until the time of necropsy.

Sample collection

Necropsy was performed on postnatal day 53 (p53), 24 h after the final injection of ChimAP for those animals that completed the experimental protocol, or sooner for those animals that appeared terminally ill. Avertin was administered intraperitoneally prior to euthanasia. Blood was collected into lithium heparin tubes by cardiac puncture. Necropsy consisted of a gross pathology examination and radiographic assessment.

Radiography and microcomputed tomography (μ CT)

Radiographic images of the entire skeletons and of partially dissected forelimbs, hindlimbs, paws and rib cage were obtained with a Faxitron MX-20DC4 (Chicago, IL, USA), using an energy of 20 kV as previously described [52,54]. Whole dissected skulls from P21 mice were fixed, then scanned at an 18 μ m isotropic voxel resolution using the eXplore Locus SP μ CT imaging system (GE Healthcare Pre-Clinical Imaging, London, ON, Canada). Measurements were taken at an operating voltage of 80 kV and 80 mA of current, with an exposure time of 1600 ms using the Parker method scan technique, which rotates the sample 180° plus a fan angle of 20°. Scans were calibrated to a hydroxyapatite phantom and 3D images were reconstructed at an effective voxel size of 18 μ m³. A fixed threshold of 1400 Hounsfield Units was used to discriminate mineralized tissue. Regions of interest (ROIs) for parietal and frontal bones were established as 1 mm in length, 1 mm in width, depth equivalent to thickness of bone and position starting at a 0.75 mm distance from sagittal and coronal sutures, as previously described [22]. Parameters of bone volume, density and structure were measured using Microview version 2.2 software (GE Healthcare Pre-Clinical Imaging, London, ON) and established algorithms [29,41]. Student's t-tests comparing quantitative results were performed to establish statistically significant differences between genotypes. μ CT bone data were analyzed and are reported in accordance with the recommendations of [4].

For dental imaging, dissected hemi-mandibles were scanned on a Scanco Medical μ CT 50 (Scanco Medical AG, Brüttisellen, Switzerland) at 10 μ m voxel size. Mandible z-stacks were exported as DICOM files and reoriented using ImageJ software (1.48r), with comparable coronal, sagittal, and transverse planes of section chosen for comparison. For quantitative analysis, mandibles were scanned on a Scanco Medical CT 35 at 6 μ m voxel size, 55 KVp, 145 mA, with 0.36° rotation step (180° angular range) and a 400 ms exposure per view. Scanco μ CT software (HP, DECwindows Motif 1.6) was used for 3D reconstruction and image viewing. After 3D reconstruction, crown, enamel, roots, and alveolar bone (of the first molar furcation region) volumes were segmented using global threshold 0.6 g/cc. Total volume (TV), bone (mineralized tissue) volume (BV), and tissue mineral density (TMD) were measured for the whole crown and separately for enamel, root dentin, and alveolar bone in the furcation region. For enamel and root, thickness was also measured.

Histological analyses

Samples of tibio-femoral joints as well as aortas, stomachs, kidneys and lungs from untreated WT age-matched control mice, vehicle-treated *Alpl*^{-/-} mice, and ChimAP1, ChimAP8 and ChimAP16 mice were cleaned, fixed in 4% paraformaldehyde/phosphate buffered saline for 3 days at 4 °C, and then transferred to 70% ethanol for storage at 4 °C. Plastic sections were prepared as described previously [53]. Von Kossa and trichrome staining was performed on plastic sections as described previously [36]. Von Kossa or Van Gieson-stained sections were scanned by ScanScopeXT system (Aperio, Vista, CA, USA), and images were analyzed by using the Bioquant Osteo software (Bioquant Osteoanalysis Co., Nashville, TN, USA). Left hemi-mandibles used for histology were fixed in Bouin's solution for 24 h, and then demineralized in AFS solution (acetic acid, formaldehyde, sodium chloride), and embedded in paraffin for serial sectioning, as described previously [11]. For picrosirius red staining, deparaffinized tissue sections were stained with 0.2% aqueous solution of phosphomolybdic acid hydrate, 0.4% Direct red 80, and 1.3% 2,4,6-trinitrophenol (Polysciences, Inc., Warrington, PA), as described previously [11]. Picrosirius red-stained sections were observed under polarized light for photomicrography.

PP_i assay

PP_i concentrations in plasma were determined by differential adsorption on activated charcoal of UDP-D-[6-3H]glucose (Amersham Pharmacia) from the reaction product 6-phospho [6-3H]gluconate, as described [18,54].

Statistics

Considering that the different doses of ChimAP resulted in different survival rates, it was not possible to compare the three treatment cohorts in an age-matched fashion. For this reason, Student t unpaired, parametric, two-tailed test was performed to compare the treated *Alpl*^{-/-} mice to the WT cohort. Differences were considered significant when $p < 0.05$. In order to compare the differences in the survival curves among the treatment cohorts, the Gehan-Breslow-Wilcoxon test was performed.

Results

Increased survival and body weight in ChimAP-treated *Alpl*^{-/-} mice

Initial pharmacokinetic studies using subcutaneous bolus injections of ChimAP revealed peak activity in plasma ($C_{\max}$) at 4 h and trough levels at 24 h ($C_{\min}$) with a half-life ($T_{1/2}$) of 6 h. Fig. 1A shows dose-dependent plasma levels attained 4 h after bolus administration into *Alpl*^{+/-} heterozygous mice and after 24 h where the residual levels of ChimAP are above the basal levels of endogenous TNAP activity in *Alpl*^{+/-} mice or WT mice. Thus, daily injections (24 h apart) were chosen for all subsequent in vivo experiments. Survival of mice receiving 8 mg/kg/day (ChimAP8) or 16 mg/kg/day (ChimAP16) of ChimAP was significantly improved compared to the vehicle-treated and the 1 mg/kg/day (ChimAP1) cohort ($p = 0.001$) (Fig. 1B). Differences were statistically significant when the Kaplan–Meier survival curves of the treated cohorts were compared against each other ($p < 0.0001$ for all comparisons). Median survival was 44, 22 and 19 days in the ChimAP8, ChimAP1 and vehicle-treated cohorts, respectively. No median survival could be calculated for the ChimAP16 cohort as the animals lived until the termination of the experiment at day 53.

Alpl^{-/-} animals weigh less than their WT littermates, starting around p7. Treatment with 1 mg/kg/day ChimAP led to statistically significant greater body weight compared to vehicle-treated mice, and a non-significant difference compared to WT littermates at 18 days of treatment (Fig. 1C). *Alpl*^{-/-} mice receiving a vitamin B6-enforced diet usually die by p18–24, therefore comparison of longer-lived ChimAP8 and ChimAP16 cohorts to vehicle-treated mice was not possible, and these were instead compared to WT mice. Animals in the ChimAP8 cohort weighed significantly less than their WT littermates from p18 (Fig. 1C) while mice in the ChimAP16 cohort showed normalization in body weight, and were undistinguishable from WT mice at p53 (Fig. 1C).

ChimAP treatment improves the skeletal phenotype of *Alpl*^{-/-} mice

Radiographs of untreated *Alpl*^{-/-} mice (Fig. 2A) demonstrated profound skeletal abnormalities including reduced tissue mineral density and fractured bones, as previously described [52]. We found prevention of the skeletal pathology in *Alpl*^{-/-} mice receiving 1 mg/kg/day ChimAP for 18 days (Fig. 2A), and the benefits were more profound in the ChimAP8 and ChimAP16 cohorts (Fig. 2B). The distal extremities displayed a normal morphology, regardless of dose, while partial prevention was observed in the spine, forelimbs, hindlimbs, and ribcage for all treatment cohorts. ChimAP8 and ChimAP16 mice also presented minor cracks or fractures in femora and tibiae and the articular surface in knees and elbows in ChimAP8 and ChimAP16 at p44 and p53, respectively does not show the smooth contour seen in WT mice (Fig. 2).

To assess the degree of prevention of osteomalacia, we performed a histomorphometric analysis of plastic-embedded undecalcified sections of hindlimbs of ChimAP-treated and WT control mice (Figs. 3A and 4A). We measured bone and osteoid volumes (Fig. 3B, C) and also analyzed serum PP_i levels (Fig. 4B, C). Consistent with earlier findings [52], von Kossa staining revealed that *Alpl*^{-/-} mice show severe defects in mineralization, thin cortical bone, reduced trabecular bone and impaired ossification centers (Fig. 3A), whereas

both ChimAP8 and ChimAP16-treated animals showed significantly better cortical bone and secondary ossification centers. Histomorphometry (Fig. 3B, C) revealed that the BV/TV ratio (Fig. 3B), both in the ChimAP8 and ChimAP16 cohorts, was still significantly lower than in the age-matched WT controls (0.0321 and 0.0302, $N = 9$), whereas the OV/BV percentage (Fig. 3C) was significantly higher for both of the treatment cohorts compared to WT controls (0.0001 and 0.0175, $N = 9$). Differences between ChimAP8 and ChimAP16 treatment cohorts were not statistically significant. Histological trichrome staining (Fig. 4A) on plastic sections of hindlimbs confirmed these findings. *Alpl*^{-/-} mice have severely reduced mineralized bone mass (green-stained regions), with deficiencies in cortical and trabecular bone marked by osteoid accumulation (red-stained regions). In contrast, 53-day-old WT controls have robust cortical bones, trabecular bone present in the secondary ossifications and little osteoid at bone surfaces. *Alpl*^{-/-} mice in the ChimAP8 and ChimAP16 cohorts display better cortical bone, especially in the ChimAP16 cohort, where no major differences were noted compared to WT mice. While trabecular bone formation in the secondary ossification centers in *Alpl*^{-/-} mice better with treatment, ChimAP8 and ChimAP16 mice still retained larger than normal regions of osteoid (red). Histological analysis of non-decalcified growth plate sections at the knee joint shows that the expansion of hypertrophic chondrocytes zone characteristically seen in *Alpl*^{-/-} mice has been prevented by the 8 mg/kg/day and 16 mg/kg/day ChimAP treatments (Fig. 5). In agreement with the skeletal findings, we found changes of the serum PP_i concentrations in all treatment cohorts. ChimAP1 animals (Fig. 4B) harbor significantly reduced PP_i levels when compared to the vehicle-treated control animals. ChimAP16 treated animals have PP_i levels that are statistically undistinguishable from that of WT littermates. These benefits to the skeleton and the changes in plasma PP_i levels did not have adverse effects on soft tissues, as we found no evidence of ectopic calcification in the aortas, stomachs, lungs or kidneys in the ChimAP1, ChimAP8 nor the ChimAP16 cohorts (Fig. S1) (Table 1).

Absence of craniofacial abnormalities in ChimAP-treated *Alpl*^{-/-} mice

Alpl^{-/-} mice feature craniofacial shape abnormalities and coronal suture fusion [22]. To determine the extent to which the craniofacial skeleton is affected by treatment in *Alpl*^{-/-} mice, we performed μ CT-based analyses of frontal and parietal cranial bones. Results at p21 show that both frontal and parietal bones of vehicle treated *Alpl*^{-/-} mice were significantly reduced in bone volume fraction, bone mineral content, bone mineral density, tissue mineral content and tissue mineral density when compared to WT or to treated *Alpl*^{-/-} mice (Table 2). In contrast, neither frontal nor parietal bones of treated *Alpl*^{-/-} mice were significantly different from those of wild type mice. Adult skulls of ChimAP16-treated *Alpl*^{-/-} mice did not appear different from WT in terms of size and shape (Fig. 6).

ChimAP treatment prevents dentoalveolar defects in *Alpl*^{-/-} mice

Ablation of *Alpl* in mice results in developmental mineralization defects in cementum, dentin, alveolar bone, and enamel [13,28,53], consistent with case reports on human subjects with HPP. Absence of acellular cementum results in loss of periodontal attachment to the tooth root surface and premature tooth exfoliation, a hallmark of HPP. ChimAP8-treated and vehicle-treated *Alpl*^{-/-} mice were compared to WT at p25–26, when molar tooth formation is near completion. Radiography and μ CT imaging revealed that, compared to controls (Fig.

7A, D), untreated *Alpl*^{-/-} mouse mandibles featured grossly hypomineralized bone, short molars with thin dentin and wide pulp chambers, and severely defective incisors (Fig. 7B, F). By histology, *Alpl*^{-/-} mouse teeth featured no acellular cementum, and alveolar bone osteoid invaded the periodontal ligament (PDL) space, leading to ankylosis and loss of a functional periodontium (Fig. 7G vs. 7E). Administration of 8 mg/kg/day ChimAP improved radiographic appearance of molar height, dentin thickness, and bone mineralization at p26, though the incisor remained defective (Fig. 7C, H). Histologically, though this dose of ChimAP did not restore acellular cementum to the root surface, the molar-associated PDL space and alveolar bone borders were better maintained (Fig. 7I).

The ChimAP16 cohort was compared with WT at p50–53 to determine effects on mature tooth structure and function. Radiography and μ CT imaging indicated reduced alveolar and interproximal bone mineralization around molar teeth in *Alpl*^{-/-} mouse mandibles, compared to controls (Fig. 7J–M). Molar tooth form and dentin appeared largely normalized in ChimAP16 mice, and this was confirmed by μ CT analysis of the first molar (Table 3). Enamel in the ChimAP16 group was not different from WT in BV/TV or TMD. Molar crowns and roots showed mild but significant decreases of 4–10% in BV/TV and TMD compared to WT, indicating dentin mineralization was not fully restored, and root thickness was decreased by 15%. Alveolar bone mineralization remained more severely defective in the ChimAP16 group, with BV/TV decreased by 27% and TMD decreased by 13%, compared to WT. Incisor teeth in treated *Alpl*^{-/-} mice also remained severely affected (Fig. 7K, M).

By histology, ChimAP16 mice featured a mix of mineralized alveolar bone and osteoid, and a reduced but maintained PDL space (Fig. 7N, P). No tooth loss was noted in the ChimAP16 group by p53, though periodontal attachment remained deficient, as evidenced by lack of cementum, PDL disorganization and detachment from the root surface, and down growth of the junctional epithelium. However, small regions of PDL attachment to the tooth were noted in association with organized PDL fibers (Fig. 7O, Q), indicating the presence of some compromised attachment that may function to retain molars.

Discussion

In this study, our goal was to evaluate the therapeutic efficacy of a soluble, non-mineral-targeting, recombinant chimeric alkaline phosphatase (ChimAP) for treatment of murine HPP. We assessed survival, body weight, enhancement of bone and dental mineralization, and prevention of craniosynostosis in ChimAP-treated *Alpl*^{-/-} mice. Previously we have shown that a mineral-targeted form of recombinant human TNAP prevented infantile HPP in a dose-dependent manner in the *Alpl*^{-/-} mouse model [31,52]. The highest dose of mineral-targeted TNAP (8.2 mg/kg/day) prevented skeletal and dental mineralization defects in *Alpl*^{-/-} mice, rescuing these tissues, where mineralization status is controlled by the extracellular P_i/PP_i ratio [33]. Here, we found that the soluble ChimAP increased survival in a dose-dependent manner, with mice receiving 16 mg/kg/day ChimAP surviving until completion of the experimental protocol (53 days) experiencing no seizures, and featuring significant enhancement of bone quality in long bones and spine, displaying normalized mineralization and no evidence of craniosynostosis in the calvarial bones, and improving

dental mineralization. These data support the hypothesis that sustained availability of non-targeted alkaline phosphatase, even one with catalytic properties different from those of TNAP is capable of providing significant therapeutic benefit in this mouse model of infantile HPP. Our studies on the characterization of ChimAP [21] have revealed a relative preference of ChimAP (as well as of its parent isozyme, IAP) for the physiological substrates in the order LPS > PLP > ATP > PP_i, while the reverse is true for TNAP, with a preference order of PP_i > PLP = ATP > LPS. This substrate preference correlates well with the known biological function of the native isozymes, where IAP is involved in maintaining gut mucosal barrier function via LPS detoxification (see [21] and references therein), while TNAP is mainly involved in the control of PP_i concentrations during skeletal mineralization and in the metabolism of Vitamin B6 [45]. Despite these catalytic differences, our work shows that ChimAP is capable of preventing many, if not all, of the manifestations of murine HPP.

TNAP plays a crucial role in promoting extracellular matrix mineralization by increasing the local availability of phosphate (P_i) needed for hydroxyapatite crystal formation, as well as by restricting the concentration of the calcification inhibitor PP_i [33]. Diminished hydrolysis of PP_i explains the impaired skeletal and dental mineralization associated with rickets and osteomalacia in individuals with HPP [45] and in *Alpl*^{-/-} mice [1,2,18]. Normalizing systemic extracellular PP_i concentrations, either via the transgenic overexpression of TNAP in liver (Murshed et al., 2005) or bone (Narisawa et al., 2013), or via administration of purified mineral-targeting TNAP [31,52] or use of viral vectors to achieve tissue-production of mineral-targeting TNAP, led to prevention of the skeletal manifestations of HPP (Murshed et al., 2005; [26,31]; Narisawa et al., 2013). Here we show that the use of a soluble ChimAP, structurally and catalytically different from TNAP, is also capable of normalizing PP_i levels, resulting in significant prevention of the skeletal and dental phenotype characteristic of *Alpl*^{-/-} mice. These benefits took place without leading to ectopic calcification of soft tissues, as we found no evidence of mineralization in the aortas, stomachs, kidneys or lungs of ChimAP-treated mice. There are contexts where use of a non-mineral-targeted form of alkaline phosphatase to treat HPP might be warranted and even desirable. First is the possibility that host antibodies may develop to prolonged treatment with mineral-targeted TNAP, as this recombinant protein contains an immunogenic deca-aspartate C-terminal sequence. To date, the clinical use of asfotase alfa has proven highly efficacious, and only low antibody titers to the drug have been measured in patients [51]. However, the possibility that with continued life-long use these antibody titers may lead to reduced efficacy cannot be discounted. The use of another isozyme of alkaline phosphatase may prove of benefit to bypass an antibody response to mineral-targeted TNAP.

A second concern arises from the possibility that, with age, treated HPP patients may develop vascular calcification, a condition that may worsen with enzyme replacement therapy. Indeed, calcification of the medial layer of arteries, recognized as an important clinical problem [6, 8], is an active process involving the chondro-osteoblastic conversion of vascular smooth muscle cells (Tyson et al., 2003), leading to the deposition of hydroxyapatite crystals within the medial layers of blood vessels [24]. The prevalence of medial vascular calcification (MVC) correlates roughly with age, occurring in 20% of young

adults, 60% of those in middle age, and 90% of the elderly [40]. Existing data implicate elevated TNAP at sites of MVC in generalized arterial calcification of infancy [37], aortic calcification due to CD73 deficiency [19] and vascular calcification in uremic rats [23], a model of chronic kidney. In all of these conditions, inappropriate calcifications are hypothesized to initiate as a result of reduced local PP_i levels, allowing deposition of bone mineral in the arteries. Recent studies have shown that up-regulation of TNAP in vascular smooth muscle cells is sufficient to cause severe MVC leading to hypertension, cardiac hypertrophy and sudden death [39]. Thus, in a certain fraction of HPP patients – those with significant residual TNAP activity – one may expect the development of medial vascular calcification from co-morbidities such as diabetes, obesity or simply aging, and the administration of mineral-targeting TNAP may aggravate this pathophysiological complication. The use of a soluble, non-mineral targeting alkaline phosphatase to continue with the sought therapeutic benefit to the skeleton may then be justified and desirable. A similar concern may be raised for nephrocalcinosis, the deposition of calcium minerals in the renal parenchyma, a prevalent condition that affected 10 of the 11 infants treated with asfotase alfa [51]. It is conceivable that the chronic administration of mineral-targeting TNAP might aggravate the nephrocalcinosis in these patients. All these possible adverse effect scenarios discussed above need to be investigated in well-controlled animal studies comparing the therapeutic benefit and potential limitations of asfotase alfa and ChimAP treatments.

Effects of ChimAP on dental mineralization were of interest because all forms of HPP negatively affect the dentition, causing premature tooth loss and lifelong dental problems [15,16,38]. The dentoalveolar complex is comprised of four distinct mineralized tissues, each with different developmental patterns and sensitivities to P_i/PP_i disturbance. ChimAP at 16 mg/kg/day rescued the *Alpl*^{-/-} mouse enamel defect [53] and partially resolved dental mineralization defects [13], perhaps in part from direct effects on dentinogenesis (shown by comparison to untreated controls at p25–26), and partly from indirect effects of increasing lifespan to allow for improvements in delayed mineralization. Acellular cementum remained defective in the ChimAP16 group, reflecting the heightened sensitivity of this tissue (compared to other mineralized tissues) to alterations in PP_i [12,13,56]. Even so, molar teeth were retained, possibly from small regions of PDL attachment to the cervical root (possibly breakthrough cementum or PDL-dentin attachment), and/or cellular cementum of the apical molar [56]. Tissues undergoing rapid turnover, including alveolar bone and the mouse continually erupting incisor, remained defective after ChimAP treatment, reflecting higher requirement of TNAP activity or local differences in modifiers of P_i/PP_i ratio. Because tooth development begins in utero in mice and humans, further studies are necessary to understand how the timing of enzyme replacement therapy will affect prevention or improvement of HPP-associated dental defects. Similar hypomineralization defects in dentin due to hypophosphatemic rickets have been shown to be amenable to postnatal treatment ([15,16]; Chaussain-Miller et al., Oral Diseases, 2007).

Overall, these pilot studies employing soluble non-mineral-targeting ChimAP support efficacy for normalizing lifespan, eliminating seizures, and preventing skeletal and dental mineralization defects associated with HPP.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was funded by grant DE12889 (JLM) from the National Institute of Dental and Craniofacial Research (NIDCR) of the National Institutes of Health (NIH), a grant from AM-Pharma (Bunnik, The Netherlands) (JLM), grant AR066110 (BLF) from the National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS)/NIH, and funding from the intramural research program of NIAMS (MJS). KG was partially supported by a fellowship from CNPq — Ciências sem fronteiras. We acknowledge the expert technical contributions of the Histology Facility at SBMRI Lake Nona, as well as Nasrin Kalantari Pour at NIAMS for dental histology. We thank Kenn Holmbeck (NIDCR/NIH) and Lyudmila Lukashova (Hospital for Special Surgery, New York, NY) for assistance in μ CT scanning and analysis of mandibles. We also thank Diana Sandoval from the Animal facility at SBMRI La Jolla for excellent help with animal breeding, maintenance and injections.

Abbreviations

ChimAP	chimeric alkaline phosphatase
PL	pyridoxal
PLP	pyridoxal phosphate
TNAP	tissue-nonspecific alkaline phosphatase
PDL	periodontal ligament
PP_i	inorganic pyrophosphate

References

1. Anderson HC, Sipe JB, Hesse L, Dhanyamraju R, Atti E, Camacho NP, et al. Impaired calcification around matrix vesicles of growth plate and bone in alkaline phosphatase-deficient mice. *Am J Pathol.* 2004; 164(3):841–7. [PubMed: 14982838]
2. Anderson HC, Harmey D, Camacho NP, Garimella R, Sipe JB, Tague S, et al. Sustained osteomalacia of long bones despite major improvement in other hypophosphatasia-related mineral deficits in tissue nonspecific alkaline phosphatase/nucleotide pyrophosphatase phosphodiesterase 1 double-deficient mice. *Am J Pathol.* 2005; 166(6):1711–20. [PubMed: 15920156]
3. Baumgartner-Sigl S, Haberlandt E, Mumm S, Scholl-Bürgi S, Sergi C, Ryan L, et al. Pyridoxine-responsive seizures as the first symptom of infantile hypophosphatasia caused by two novel missense mutations (c.677T>C, p.M226T; c.1112C>T, p. T371I) of the tissue-nonspecific alkaline phosphatase gene. *Bone.* 2007; 40:1655–61. [PubMed: 17395561]
4. Bouxsein ML, Boyd SK, Christiansen BA, Guldberg RE, Jepsen KJ, Müller R. Guidelines for assessment of bone microstructure in rodents using micro-computed tomography. *J Bone Miner Res.* 2010; 25(7):1468–86. [PubMed: 20533309]
5. Cahill RA, Wenkert D, Perlman SA, Steele A, Coburn SP, Mcalister WH, et al. Infantile hypophosphatasia: transplantation therapy trial using bone fragments and cultured osteoblasts. *J Clin Endocrinol Metab.* 2007; 92:2923–30. [PubMed: 17519318]
6. Chen NX, Moe SM. Arterial calcification in diabetes. *Curr Diab Rep.* 2003; 3:28–32. [PubMed: 12643143]
7. Chodirker BN, Coburn SP, Seargeant LE, Whyte MP, Greenberg CR. Increased plasma pyridoxal-5'-phosphate levels before and after pyridoxine loading in carriers of perinatal/infantile hypophosphatasia. *J Inher Metab Dis.* 1990; 13:891–6. [PubMed: 2079838]

8. Doherty TM, Fitzpatrick LA, Inoue D, Qiao JH, Fishbein MC, Detrano RC, et al. Molecular, endocrine, and genetic mechanisms of arterial calcification. *Endocr Rev.* 2004; 25:629–72. [PubMed: 15294885]
9. Fauvert D, Brun-Heath I, Lia-Baldini A-S, Bellazi L, Taillandier A, Serre J-L, et al. Mild forms of hypophosphatasia mostly result from dominant negative effects of severe alleles or from compound heterozygosity for severe and moderate alleles. *BMC Med Genet.* 2009; 10:51. <http://dx.doi.org/10.1186/1471-2350-10-51>. [PubMed: 19500388]
10. Fedde KN, Blair L, Silverstein J, Coburn SP, Ryan LM, Weinstein RS, et al. Alkaline phosphatase knockout mice recapitulate the metabolic and skeletal defects of infantile hypophosphatasia. *J Bone Miner Res.* 1999; 14:2015–26. [PubMed: 10620060]
11. Foster BL. Methods for studying tooth root cementum by light microscopy. *Int J Oral Sci.* 2012; 4(3):119–28. [PubMed: 22996273]
12. Foster BL, Nagatomo KJ, Bamashmou SO, Tompkins KA, Fong H, Dunn D, et al. The progressive ankylosis protein regulates cementum apposition and extracellular matrix composition. *Cells Tissues Organs.* 2011; 194(5):382–405. [PubMed: 21389671]
13. Foster BL, Nagatomo KJ, Nociti FH, Fong H, Dunn D, Tran AB, et al. Central role of pyrophosphate in acellular cementum formation. *PLoS One.* 2012; 7(6):e38393. [PubMed: 22675556]
14. Foster BL, Nagatomo KJ, Tso HW, Tran AB, Nociti FH Jr, Narisawa S, et al. Tooth root dentin mineralization defects in a mouse model of hypophosphatasia. *J Bone Miner Res.* 2013; 28(2): 271–82. [PubMed: 22991301]
15. Foster BL, Nociti FH Jr, Somerman MJ. The rachitic tooth. *Endocr Rev.* 2014; 35(1):1–34. [PubMed: 23939820]
16. Foster BL, Ramnitz MS, Gafni RI, Burke AB, Boyce AM, Lee JS, et al. Rare bone diseases and their dental, oral, and craniofacial manifestations. *J Dent Res.* 2014; 93(7 Suppl):7S–19S. [PubMed: 24700690]
17. Henthorn PS, Raducha M, Fedde KN, Lafferty MA, Whyte MP. Different missense mutations at the tissue-nonspecific alkaline phosphatase gene locus in autosomal recessively inherited forms of mild and severe hypophosphatasia. *Proc Natl Acad Sci U S A.* 1992; 89:9924–8. [PubMed: 1409720]
18. Hessle L, Johnson KA, Anderson HC, Narisawa S, Sali A, Goding JW, et al. Tissue-nonspecific alkaline phosphatase and plasma cell membrane glycoprotein-1 are central antagonistic regulators of bone mineralization. *Proc Natl Acad Sci U S A.* 2002; 99(14):9445–9. [PubMed: 12082181]
19. St Hilaire C, Ziegler SG, Markello TC, et al. NT5E mutations and arterial calcifications. *N Engl J Med.* 2011; 364:432–42. [PubMed: 21288095]
20. Huesa C, Yadav MC, Finnillä MA, Goodyear SR, Robins SP, Tanner KE, et al. PHOSPHO1 is essential for mechanically competent mineralization and the avoidance of spontaneous fractures. *Bone.* 2011; 48(5):1066–74. [PubMed: 21272676]
21. Kiffer-Moreira T, Sheen CR, Gasque K, Bolean M, Ciancaglini P, Van Elsas A, et al. Catalytic signature of a heat-stable, chimeric human alkaline phosphatase with therapeutic potential. *PLoS One.* 2014; 9(2):e89374. <http://dx.doi.org/10.1371/journal.pone.0089374>. [PubMed: 24586729]
22. Liu J, Nam HK, Campbell C, Gasque KC, Millán JL, Hatch NE. Tissue-nonspecific alkaline phosphatase deficiency causes abnormal craniofacial bone development in the *Alpl*(^{-/-}) mouse model of infantile hypophosphatasia. *Bone.* 2014; 67:81–94. [PubMed: 25014884]
23. Lomashvili KA, Garg P, Narisawa S, Millán JL, O'Neill WC. Upregulation of alkaline phosphatase and pyrophosphate hydrolysis: potential mechanism for uremic vascular calcification. *Kidney Int.* 2008; 73(9):1024–30. [PubMed: 18288101]
24. Lyemere VP, Proudfoot D, Weissberg PL, Shanahan CM. Vascular smooth muscle cell phenotypic plasticity and the regulation of vascular calcification. *J Intern Med.* 2006; 260:199–210.
25. Markello TC, Pak LK, St Hilaire C, Dorward H, Ziegler SG, Chen MY, et al. Vascular pathology of medial arterial calcification in NT5E deficiency: implications for the role of adenosine in pseudoxanthoma elasticum. *Mol Genet Metab.* 2011; 103:44–50. [PubMed: 21371928]

26. Matsumoto T, Miyake K, Yamamoto S, Orimo H, Miyake N, Odagaki Y, et al. Rescue of severe infantile hypophosphatasia mice by AAV-mediated sustained expression of soluble alkaline phosphatase. *Hum Gene Ther.* 2011; 22(11):1355–64. [PubMed: 21388343]
27. Meyer JL. Can biological calcification occur in the presence of pyrophosphate? *Arch Biochem Biophys.* 1984; 231:1–8. [PubMed: 6326671]
28. McKee MD, Nakano Y, Masica DL, Gray JJ, Lemire I, Heft R, et al. Enzyme replacement therapy prevents dental defects in a model of hypophosphatasia. *J Dent Res.* 2011; 90(4):470–6. [PubMed: 21212313]
29. Meganck JA, Kozloff KM, Thornton MM, Broski SM, Goldstein SA. Beam hardening artifacts in micro-computed tomography scanning can be reduced by X-ray beam filtration and the resulting images can be used to accurately measure BMD. *Bone.* 2009; 45(6):1104–16. [PubMed: 19651256]
30. Millán, JL. From biology to applications in medicine and biotechnology. Weinheim, Germany: Wiley-VCH Verlag GmbH & Co; 2006. Mammalian alkaline phosphatases; p. 1-322.
31. Millán JL, Narisawa S, Lemire I, Loisel TP, Boileau G, Leonard P, et al. Enzyme replacement therapy for murine hypophosphatasia. *J Bone Miner Res.* 2008; 23:777–87. [PubMed: 18086009]
32. Millán JL, Plotkin H. Hypophosphatasia — pathophysiology and treatment. *AAOMM.* 2012; 8(3): 164–82.
33. Millán JL. The role of phosphatases in the initiation of skeletal mineralization. *Calcif Tissue Int.* 2013; 93(4):299–306. <http://dx.doi.org/10.1007/s00223-012-9672-8> [ePub ahead of print. Epub 2012/11/28]. [PubMed: 23183786]
34. Mornet E, Taillandier A, Peyramaure S, Kaper F, Muller F, Brenner R, et al. Identification of fifteen novel mutations in the tissue-nonspecific alkaline phosphatase (TNSALP) gene in European patients with severe hypophosphatasia. *Eur J Hum Genet.* 1998; 6:308–14. [PubMed: 9781036]
35. Narisawa S, Millán JL, Wennberg C. Abnormal vitamin B6 metabolism in alkaline phosphatase knock-out mice causes multiple abnormalities, but not the impaired bone mineralization. *J Pathol.* 2001; 193:125–33. [PubMed: 11169525]
36. Narisawa S, Fröhlander N, Millán JL. Inactivation of two mouse alkaline phosphatase genes and establishment of a model of infantile hypophosphatasia. *Dev Dyn.* 1997; 208:432–46. [PubMed: 9056646]
37. Narisawa S, Harmey D, Yadav MC, O'Neill WC, Hoylaerts MF, Millán JL. Novel inhibitors of alkaline phosphatase suppress vascular smooth muscle cell calcification. *J Bone Miner Res.* 2007; 22:1700–10. [PubMed: 17638573]
38. Reibel A, Manière M, Clauss F, Droz D, Alembik Y, Mornet E, et al. Orodonal phenotype and genotype findings in all subtypes of hypophosphatasia. *Orphanet J Rare Dis.* 2009; 4:6. [PubMed: 19232125]
39. Sheen CR, Kuss P, Narisawa S, Yadav MC, Nigro J, Wang W, et al. Pathophysiological role of vascular smooth muscle alkaline phosphatase in medial artery calcification. *J Bone Miner Res.* 2015 Revision submitted.
40. Towler DA, Demer LL. Thematic series on the pathobiology of vascular calcification: an introduction. *Circ Res.* 2011; 108:1378–80. [PubMed: 21617134]
41. Umoh JU, Sampaio AV, Welch I, Pitelka V, Goldberg HA, Underhill TM, et al. In vivo micro-CT analysis of bone remodeling in a rat calvarial defect model. *Phys Med Biol.* 2009; 54(7):2147–61. [PubMed: 19287088]
42. Waymire KG, Mahuren JD, Jaje JM, Guilarte TR, Coburn SP, MacGregor GR. Mice lacking tissue non-specific alkaline phosphatase die from seizures due to defective metabolism of vitamin B-6. *Nat Genet.* 1995; 11:45–51. [PubMed: 7550313]
43. Weiss MJ, Cole DE, Ray K, Whyte MP, Lafferty MA, Mulivor RA, et al. A missense mutation in the human liver/bone/kidney alkaline phosphatase gene causing a lethal form of hypophosphatasia. *Proc Natl Acad Sci U S A.* 1988; 85:7666–9. [PubMed: 3174660]
44. Weninger M, Stinson RA, Plenk H Jr, Böck P, Pollak A. Biochemical and morphological effects of human hepatic alkaline phosphatase in a neonate with hypophosphatasia. *Acta Paediatr Scand Suppl.* 1989; 360:154–60. [PubMed: 2642253]

45. Whyte, M. Hypophosphatasia. In: Glorieux, F.; Jueppner, H.; Pettifor, J., editors. Pediatric bone. San Diego, CA: Elsevier (Academic Press); 2012. p. 771-94.
46. Whyte MP, Kurtzberg J, Mcalister WH, Mumm S, Podgornik MN, Coburn SP, et al. Marrow cell transplantation for infantile hypophosphatasia. *J Bone Miner Res.* 2003; 18:624–36. [PubMed: 12674323]
47. Whyte MP, Mahuren JD, Vrabel LA, Coburn SP. Markedly increased circulating pyridoxal-5'-phosphate levels in hypophosphatasia. Alkaline phosphatase acts in vitamin B6 metabolism. *J Clin Invest.* 1985; 76:752–6. [PubMed: 4031070]
48. Whyte MP, Habib D, Coburn SP, Tecklenburg F, Ryan L, Fedde KN, et al. Failure of hyperphosphatasemia by intravenous infusion of purified placental alkaline phosphatase (ALP) to correct severe hypophosphatasia: evidence against a role for circulating ALP in skeletal mineralization. *J Bone Miner Res.* 1992; 7(Suppl 1):S155.
49. Whyte MP, Valdes R Jr, Ryan LM, McAlister WH. Infantile hypophosphatasia: enzyme replacement therapy by intravenous infusion of alkaline phosphatase-rich plasma from patients with Paget bone disease. *J Pediatr.* 1982; 101:379–86. [PubMed: 7108657]
50. Whyte MP, McAlister WH, Patton LS, Magill HL, Fallon MD, Lorentz WB Jr, et al. Enzyme replacement therapy for infantile hypophosphatasia attempted by intravenous infusions of alkaline phosphatase-rich Paget plasma: results in three additional patients. *J Pediatr.* 1984; 105:926–33. [PubMed: 6502342]
51. Whyte MP, Greenberg CR, Salman NJ, Bober MB, McAlister WH, Wenkert D, et al. Enzyme-replacement therapy in life-threatening hypophosphatasia. *N Engl J Med.* 2012; 366(10):904–13. [PubMed: 22397652]
52. Yadav MC, Lemire I, Leonard P, Boileau G, Blond L, Beliveau M, et al. Dose response of bone-targeted enzyme replacement for murine hypophosphatasia. *Bone.* 2011; 49(2):250–6. [PubMed: 21458605]
53. Yadav MC, de Oliveira RC, Foster BL, Fong H, Cory E, Narisawa S, et al. Enzyme replacement prevents enamel defects in hypophosphatasia mice. *J Bone Miner Res.* 2012; 27(8):1722–34. [PubMed: 22461224]
54. Yadav MC, Huesa C, Narisawa S, Hoylaerts MF, Moreau A, Farquharson C, et al. Ablation of osteopontin improves the skeletal phenotype of *Phospho1*^{-/-} mice. *J Bone Miner Res.* 2014; 29:2369–81. <http://dx.doi.org/10.1002/jbmr.2281>. [PubMed: 24825455]
55. Zurutuza L, Muller F, Gibrat JF, Taillandier A, Simon-Bouy B, Serre J-L, et al. Correlations of genotype and phenotype in hypophosphatasia. *Hum Mol Genet.* 1999; 8:1039–46. [PubMed: 10332035]
56. Zweifler LE, Patel MK, Nociti FH Jr, Wimer H, Millán JL, Somerman MJ, et al. Counter-regulatory phosphatases TNAP and NPP1 temporally regulate tooth root cementogenesis. *Int J Oral Sci.* 2014 In press.

Fig. 1.

Pharmacokinetics and improvement of survival and body weight of *Alpl*^{-/-} mice by ChimAP treatment. A) Subcutaneous injections of 1 mg/kg, 8 mg/kg and 16 mg/kg ChimAP into *Alpl*^{+/-} heterozygous mice led to dose-dependent peak plasma AP levels after 4 h and trough plasma AP levels at 24 h that were slightly higher than endogenous TNAP activity levels in both heterozygous and WT mice. Dosing once a day was deemed adequate for the in vivo efficacy studies. B) Kaplan–Meier curves show that ChimAP16 mice survived to the end of the experiment. Average survival for Vehicle, ChimAP1, and ChimAP8 mice was p19, p22 and p42.5, respectively. C) *Alpl*^{-/-} vehicle treated mice are lighter than WT littermates; ChimAP1 treatment improves body weight close to WT at p18. Vehicle treated mice do not survive longer and ChimAP8 treated mice are still lighter than WT littermates at p53. Whereas ChimAP16 treated mice present a body weight not significantly different from their WT littermates at p53.

Fig. 2.

ChimAP treatment improves the skeletal phenotype of *Alpl*^{-/-} mice. Radiographs of spine, forelimb, rib cage, hindlimb and paws from *Alpl*^{-/-} mice treated with vehicle, ChimAP1, ChimAP8, ChimAP16, and untreated WT controls (magnification 5×). A) *Alpl*^{-/-} mice display a severe hypomineralization (arrows) in vertebrae, long bones and rib cage. Secondary ossification centers are missing in *Alpl*^{-/-} mice (asterisk). Treatment with ChimAP1 slightly corrects that phenotype (arrowheads) compared to untreated WT at p18. B) Treatment with ChimAP8 and ChimAP16 clearly corrects the bone phenotype in vertebrae, long bones and rib cage. Specifically the distal most extremities are improved as the development of the secondary ossification centers in the metatarsal region demonstrates (arrowhead).

Fig. 3.

Improved mineralization in ChimAP-treated *Alpl*^{-/-} mice. A) Histological analysis of femora of vehicle treated *Alpl*^{-/-} (p18), ChimAP8 (p51), ChimAP16 (p53), and untreated WT mice (p53). Von Kossa staining revealed better bone mineralization with increased dosages ChimAP8 and ChimAP16. Secondary ossification centers and the cortical bones are strikingly better (black) ChimAP-treated *Alpl*^{-/-} mice compared to vehicle-treated *Alpl*^{-/-} mice. There is less trabecular bone in the ChimAP8 and ChimAP16 mice compared to WT, but increased mineralization in the trabecular region as well as more osteoid (pink). B/C) Analysis of BV/TV and OV/BV for ChimAP8 and ChimAP16 demonstrated less bone volume and higher osteoid volume than age-matched controls. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 4.

Improvement of osteomalacia and plasma PP_i levels in ChimAP-treated *Alpl*^{-/-} mice. A) Histological analysis of femora of vehicle treated *Alpl*^{-/-} (p18) (n = 3), ChimAP8 (p51) (n = 3), ChimAP16 p53 (n = 3), and age-matched untreated sibling p53 WT control mice (n = 3). Goldner's Trichrome staining of the femora sections show severe osteomalacia in *Alpl*^{-/-}, and improved mineralization (green) in the cortical area and in the secondary ossification with increased dosages ChimAP8 and ChimAP16. Presence of enlarged areas of osteoid (red), suggests deposition of bone, but not yet mineralized. B/C) PP_i concentrations in the plasma of *Alpl*^{-/-} mice receiving ChimAP1 (n = 5), ChimAP16 (n = 5), and untreated age-matched sibling WT (n = 5) control mice. ChimAP1 treatment leads to a significant reduction of the elevated PP_i levels in *Alpl*^{-/-} mice at p18. At p53 ChimAP16 treatment resulted in a correction of plasma PP_i levels comparable to WT controls. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 5.

Prevention of hypertrophic zone expansion in the growth plates of ChimAP-treated *Alpl*^{-/-} mice. Histological analysis using Goldner's Trichrome staining of non-decalcified growth plate sections obtained at the knee joint of vehicle-treated *Alpl*^{-/-} (p20), ChimAP8 (p51), ChimAP16 (p53), and untreated age-matched sibling WT control mice (p53), shown in 10× (upper panels) and 20× magnification (lower panels). HZ: hypertrophic zone.

Fig. 6.

Absence of craniofacial defects in ChimAP-treated *Alpl*^{-/-} mice. μ CT isosurface images of WT (A, D), vehicle-treated *Alpl*^{-/-} (B, E) and ChimAP16 (C, F) mouse skulls at p21 and p53, respectively. Neither frontal nor parietal bones of treated *Alpl*^{-/-} mice were significantly different from those of WT mice. Adult skulls of ChimAP16-treated *Alpl*^{-/-} mice did not appear different from WT in terms of size and shape.

Fig. 7. ChimAP treatment prevents dentoalveolar pathology in *Alpl*^{-/-} mice. Compared to (A) radiography and (D) μ CT analysis of wild-type controls at p25–26, (B, F) untreated *Alpl*^{-/-} mouse mandibles feature hypomineralized and reduced alveolar bone (AB), short molars (M1, M2, and M3) with thin dentin (DE) and wide pulp chambers, and defective (white asterisk) incisor (INC) dentin. Compared to (E) histology of control periodontal tissues, (G) *Alpl*^{-/-} mice exhibit no acellular cementum (AC), and alveolar bone osteoid invades the PDL space, creating bone-tooth ankylosis (asterisk). (C, H) ChimAP8-treated *Alpl*^{-/-} mice show improved radiographic appearance of molar height, dentin thickness, and bone mineralization, though the incisor teeth remain defective. (I) Histologically ChimAP does not restore acellular cementum to the root surface. Compared to (J, L) controls at p53, (K, M) ChimAP16-treated *Alpl*^{-/-} mice exhibit reduced alveolar bone mineralization around molar teeth. Molar form and mineralization appear relatively normal in treated *Alpl*^{-/-} mice,

while incisor teeth remain severely affected on the root analogue (white asterisk). Compared to histology of (N) WT control molars, (P) ChimAP16-treated *Alpl*^{-/-} mice feature a mix of mineralized alveolar bone and osteoid (asterisks), and a reduced but maintained PDL space. Poor periodontal attachment is evidenced by lack of cementum, PDL disorganization and detachment, and down growth of the junctional epithelium. Small regions of PDL attachment (chevron) to the tooth were noted. Compared to the strong and parallel organization of PDL collagen fibers in (O) control tissues, indicated by picosirius staining under polarized light, (Q) ChimAP16-treated *Alpl*^{-/-} mice feature a less organized PDL, though regions of organization and attachment exist adjacent to breakthrough areas of tooth root attachment (white chevron).

Table 1

Length of tibiae, femora, radii and humeri of vehicle, ChimAP1, ChimAP8 and ChimAP16 treatment cohorts compared to their age-matched untreated WT littermates.

Bone ^a	Genotype		p	n
	<i>Alpl</i> ^{-/-}	WT		
Tibia				
ChimAP 16	1.65 ± 0.026	1.73 ± 0.062	ns	10
ChimAP 8	1.60 ± 0	1.76 ± 0.057	0.0001	7
ChimAP 1	1.02 ± 0.050	1.31 ± 0.045	0.0001	8
Untreated	1.27 ± 0.040	1.47 ± 0.017	0.0012	7
Femur				
ChimAP 16	1.32 ± 0.029	1.20 ± 0.045	0.0291	10
ChimAP 8	1.04 ± 0.049	1.22 ± 0.045	0.0125	10
ChimAP 1	0.87 ± 0.025	0.95 ± 0.034	0.0204	12
Untreated	0.91 ± 0.074	1.08 ± 0.017	ns	15
Radius				
ChimAP 16	1.31 ± 0.044	1.41 ± 0.042	ns	8
ChimAP 8	1.14 ± 0.028	1.23 ± 0.013	0.0027	7
ChimAP 1	0.96 ± 0.419	1.11 ± 0.022	0.002	8
Untreated	1.06 ± 0.048	1.28 ± 0.031	0.011	7
Humerus				
ChimAP 16	1.07 ± 0.031	1.09 ± 0.031	ns	8
ChimAP 8	1.01 ± 0.040	1.10 ± 0.004	0.0070	7
ChimAP 1	0.80 ± 0.027	0.91 ± 0.023	0.0089	8
Untreated	0.84 ± 0.037	0.93 ± 0.021	0.0335	7

ns: no statistical significance between genotypes.

^aBone samples from *Alpl*^{-/-} and WT littermate controls were obtained at different ages for each treatment cohort. ChimAP16 at 53 days, ChimAP8 at 44 days, ChimAP1 at 24 days and Vehicle-treated at 18 days. Therefore each treatment group also includes data for age-matched untreated WT littermates.

µCT analyses of cranial bones. Frontal and parietal bones were analyzed in WT, untreated *Alpl*^{-/-} (vehicle), and ChimAP16-treated *Alpl*^{-/-} mice at p21. Values are reported as means ± SD.

Table 2

	Bone volume fraction	Bone mineral content (mg)	Bone mineral density (mg/cc)	Tissue mineral content (mg)	Tissue mineral density (mg/cc)
<i>Frontal</i>					
<i>Alpl</i> ^{-/-}	0.41 ± 0.06*	0.008 ± 0.001*	464 ± 27*	0.004 ± 0.001*	595 ± 28*
ChimAP16	0.72 ± 0.15	0.018 ± 0.005	615 ± 84	0.015 ± 0.006	697 ± 57
WT	0.70 ± 0.12	0.019 ± 0.008	615 ± 79	0.016 ± 0.008	709 ± 56
<i>Parietal</i>					
<i>Alpl</i> ^{-/-}	0.52 ± 0.05*	0.018 ± 0.001*	494 ± 21*	0.005 ± 0.001*	597 ± 12*
ChimAP16	0.72 ± 0.08	0.018 ± 0.007	608 ± 74	0.015 ± 0.006	691 ± 61
WT	0.76 ± 0.10	0.020 ± 0.007	645 ± 73	0.017 ± 0.007	726 ± 51

Three comparisons were run:

Alpl^{-/-} vehicle vs. *Alpl*^{-/-} ChimAP16 (treatment comparison) — statistical differences were found (p < 0.05).

Alpl^{-/-} vehicle vs. WT (genotype comparison) — statistical differences were found (p < 0.05).

Alpl^{-/-} ChimAP16 vs. WT — no statistical differences were found.

* Indicates statistical significance between genotypes and between treatment cohorts (p < 0.05).

Table 3

MicroCT analysis of dentoalveolar tissues. First mandibular molars and associated alveolar bone were compared at p50-53 in WT (n = 5) and *Alpl*^{-/-} mice treated with ChimAP16 (n = 4). Values are reported as means ± SD.

	TV (mm ³)	BV (mm ³)	BV/TV (%)	TMD (g HA/cm ³)	Thickness (μm)
<i>Enamel</i>					
WT	0.25 ± 0.03	0.24 ± 0.02	98.77 ± 0.52	1.69 ± 0.05	70.2 ± 0.5
ChimAP16	0.20 ± 0.02 *	0.20 ± 0.02 *	98.97 ± 0.27	1.68 ± 0.03	63.0 ± 0.5
<i>Crown</i>					
WT	0.61 ± 0.04	0.56 ± 0.03	91.06 ± 0.64	ND	ND
ChimAP16	0.53 ± 0.03 *	0.46 ± 0.03 *	86.92 ± 0.69 *	ND	ND
<i>Root</i>					
WT	0.65 ± 0.05	0.56 ± 0.05	85.84 ± 0.74	1.07 ± 0.01	140.4 ± 8.4
ChimAP16	0.56 ± 0.06	0.44 ± 0.04 *	77.27 ± 2.37 *	1.02 ± 0.01 *	121.5 ± 1.7 *
<i>Alveolar bone</i>					
WT	0.28 ± 0.01	0.16 ± 0.02	57.40 ± 7.52	1.01 ± 0.02	ND
ChimAP16	0.22 ± 0.03 *	0.07 ± 0.01 *	34.56 ± 4.71 *	0.85 ± 0.02 *	ND

ND = Not determined.

* p < 0.05 by independent samples t-test.